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Redesign of Retrovirus Packaging Cell Lines To Avoid Recombination Leading to Helper Virus Production

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Retrovirus vectors can be made in the absence of helper virus by using retrovirus packaging cell lines. Helper-free virus is critical for a variety of gene transfer studies. The most useful packaging cell lines contain helper virus DNA from which the signal required for packaging of the viral RNA genome into virions has been deleted. However, we showed that the ability to package virus is conferred at very low frequency to cells infected with virus from these packaging cell lines, presumably by low-frequency transmission of the deleted virus genome. In addition, these packaging cell lines can interact with some retroviral vectors to yield replication-competent virus. We constructed packaging cell lines containing helper virus DNA that had several alterations in addition to deletion of the packaging signal. The new packaging cells retained the useful features of previously available lines but did not yield helper virus after introduction of any of the vectors tested, and transfer of the packaging function was not detected.

Retroviruses have become important tools for efficient transfer of genes into eucaryotic cells. A major part of their utility is due to the availability of retrovirus packaging cell lines (3, 13, 15, 24, 27) which allow production of replication-defective retrovirus vectors in the absence of helper virus. Such vectors infect and integrate into cells but cannot replicate and spread. These properties make possible a variety of studies in which virus spread would make interpretation of results difficult or impossible. For example, helper-free vectors allow study of hemopoietic cell lineage relationships in intact animals owing to the presence of one to a few unique viral integration sites in each infected cell (5, 11). The absence of helper virus prevents the occurrence of new integration events. An additional important use of retroviruses may be in human gene therapy (1), and these viruses must be helper free to avoid helper-induced disease or virus spread outside the treated patient.

However, there are several problems with currently available retrovirus packaging lines. Some have limited host ranges (13, 27) or produce only low titers of retroviral vectors (24). Both of the high-titer, wide-host-range packaging cell lines currently available are nearly identical in construction (3, 15) and rely on viral protein synthesis from a provirus almost identical with a helper virus, except that the signal for packaging of viral RNA has been deleted. Recently, however, low-level transmission of a retroviral vector lacking a packaging signal has been reported (12); thus, one might expect that the deleted viral genome in these retrovirus packaging cell lines would also be transmitted at low frequency. We demonstrate that transfer of the packaging function does indeed occur. In addition, it has previously been shown that certain retroviral vectors interact with these packaging cells to produce high levels of helper virus, presumably following recombination between the vector and packaging system (16).

We tested several new designs for packaging cell lines and found that one of these, which contains several mutations in addition to deletion of the packaging signal, is not subject to the problems described above; we did not detect helper virus production or packaging function transfer using this line, even after introduction of vectors which caused helper virus production from previously described packaging lines.

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MATERIALS AND METHODS

Cell culture. Cells were grown in Dulbecco modified Eagle medium with high glucose (4.5 g/liter) supplemented with 10% calf serum (Psi-2 cells) or 10% fetal bovine serum (all other cell lines). Previously described cell lines included NIH 3T3 TK$^-$ (28), PA12 (15), Psi-2 (13), and 208F (19). The PA317 cell line generated by this study is available from the American Type Culture Collection (no. CRL 9078). Cells were free of *Mycoplasma* sp. as determined by using the DNA stain Hoechst 33258 (22).

Virus assay. For assay of virus carrying selectable markers, recipient cells were seeded at 5×10^5 per 60-mm dish on day 1. On day 2 the medium was changed to medium containing 4 μg of polybrene per ml, and test virus samples were added. Unless otherwise indicated, virus was harvested by exposing culture medium to confluent dishes of virus-producing cells for 16 h, removing the medium, and subjecting the medium to centrifugation at 3,000 × *g* for 5 min to remove cells and debris. On day 3 the cells were split 1:10 into selective medium; 10^{-7} M methotrexate for virus expressing a mutant dihydrofolate reductase (DHFR), 2 mg of G-418 (about 50% active) per ml for Neo virus, or HAT selective medium (30 μM hypoxanthine, 1 μM amethopterin, 20 μM thymidine) for hypoxanthine-guanine phosphoribosyltransferase (HPRT) virus. Colonies were stained and counted on day 9. Helper virus was measured by using the S^+L^- assay as previously described (15).

Generation and testing of packaging cell lines. Clonal cell lines containing the packaging constructs were made by transfecting (4, 7) NIH 3T3 TK$^-$ cells, seeded the day before at 5×10^5 per 60-mm dish, with 10 μg of packaging construct DNA and 0.1 μg of the herpes simplex virus thymidine kinase gene carried as a *Bam*HI fragment in pBR322 (2). The cells were grown in HAT selective medium and resultant TK$^+$ colonies were isolated with cloning rings. Clones were screened for their ability to package a retroviral vector containing the selectable marker HPRT as follows. On day one, cells to be tested were seeded at 5×10^5 cells per 60-mm dish. On day 2, the cells were transfected with 10 μg of

HPRT$^-$ virus plasmid pLPL2 (see Fig. 2). On day 3, the transfected cells were fed. On day 4, medium from the transfected cells was removed and centrifuged at 3,000 × *g* for 5 min to remove cells and debris, and samples were analyzed for HPRT virus by using HPRT$^-$ rat 208F cells and for helper virus by using the S$^+$L$^-$ assay.

Cocultivation assay for packaging function transfer. NIH 3T3 cells nonproductively infected with N2 Neo virus (NIH 3T3-N2 cells) were made by infecting NIH 3T3 cells with helper-free N2 virus from PA317-N2 cells (see Table 3) and selecting G-418-resistant cells. Amphotropic murine leukemia virus (AM-MLV) (see Fig. 1) infection of clonal NIH 3T3-N2 cells resulted in production of 10^6 to 10^7 Neo virus per ml of medium exposed to the cells, showing that Neo virus could be efficiently rescued. Cell lines to be tested for production of virions containing the packaging system were seeded at 10^6 cells per 60-mm dish on day 1. On day 2, NIH 3T3-N2 cells were seeded at 10^5 per 60-mm dish, and the test cells were fed. In the morning of day 3, 12 h after the test cells had been fed, medium from the test cells was removed and centrifuged at 3,000 × *g* for 5 min to remove cells and debris, and the NIH 3T3-N2 cells were infected with 1-ml samples in the presence of 4 μg of polybrene per ml. The test cells were fed, and the NIH 3T3-N2 cells were again infected 12 h later. Infected NIH 3T3-N2 cells were fed on day 4, and on day 5 they were trypsinized, divided into two samples, and added to 60-mm dishes containing 8 × 10^5 208F cells each. On day 6, the cocultivated cells were confluent and were trypsinized and divided 1:10. On day 9, after a total of 4 days of cocultivation, the cells were split 1:10 into 100-mm dishes in medium containing 2 mg of G-418 (ca. 50% active) per ml and 20 μM 6-thioguanine. Cells were fed with selective medium every 3 days, and colonies were stained and counted on day 14. NIH 3T3-N2 cells die in medium containing 6-thioguanine, and 208F cells die in G-418, so the only cells that survived were 208F cells infected with Neo virus rescued from the NIH 3T3-N2 cells.

RESULTS

Construction of mutants. Elements required for retrovirus replication can be divided into *cis*- and *trans*-acting factors. The *trans*-acting factors include proteins encoded by the viral genome, which are required for encapsidation of the viral RNA, entry of virions into cells, reverse transcription of the viral genome, and integration of the DNA form of the virus into host DNA. The *cis*-acting factors include signals present in the viral RNA which interact with these proteins and other factors during virus replication. To make a retrovirus packaging cell line with the lowest propensity for generating replication-competent virus, we made alterations in the viral genome which should interfere with *cis*-acting elements while preserving production of *trans*-acting factors. A cell line containing such an altered viral genome should not transmit this virus but would transmit other viral RNAs containing the proper *cis*-acting elements, including retroviral vectors designed to carry foreign genes.

We chose the replication-competent amphotropic retrovirus AM-MLV (Fig. 1) for production of mutant viruses (Fig. 1) because of the broad host range of this virus, which includes mice, rats, chickens, cats, dogs, monkeys, and humans (8, 15, 20). The *trans*-acting factors encoded by this murine retrovirus include the *gag* and *pol* proteins, which are translated from unspliced viral genomic RNA, and the *env* protein, which is translated from a spliced message. Previous work has shown that a region between the 5′ splice site and the initiator codon of the *gag* protein is required for efficient packaging of retroviral RNA into virions (13, 23). This packaging signal was removed from pAM-MLV to make pPAM. In pPAM2 the packaging signal and viral sequences 3′ of the *env* protein terminator codon have been removed. The simian virus 40 late-region polyadenylation signal was added at the 3′ end to provide for polyadenylation of viral mRNAs. The deletion at the 3′ end of the virus removes the site for initiation of second-strand DNA synthesis and the 3′ R region that is required for translocation of reverse transcriptase during first-strand DNA synthesis (26). In addition to these changes, pPAM3 has a deletion of the 5′ end of the 5′ long terminal repeat (LTR); thus, a proper integration signal cannot be made from the remaining sequences and this should prevent virus integration (18). In pPAM4, all of the above deletions have been made and the 3′ end of the 5′ LTR and the tRNA primer-binding site have been removed. The viral promoter and the splice donor used to make the *env* mRNA were left intact in pPAM4. Deletion of the tRNA-binding site should inhibit first-strand DNA synthesis from the viral genome (26).

Generation of retrovirus packaging cell lines. We introduced the DNA constructs depicted in Fig. 1 into NIH 3T3 TK$^-$ cells by cotransfection using the herpes simplex virus thymidine kinase gene (2) as a selectable marker. TK$^+$ colonies were isolated and screened for production of replication-competent virus by using the S$^+$L$^-$ assay and for their ability to package a standard retrovirus vector containing HPRT (Fig. 2). We were unable to detect helper virus production from any of the clones analyzed (>70 clones) by using the S$^+$L$^-$ assay with a limit of sensitivity of 1 virus per ml. At least 50% of the clonal cell lines made with any one of the packaging constructs were able to package HPRT virus (Table 1), suggesting that rearrangement of the transfected DNA was not required to obtain such lines. Roughly equivalent virus titers were obtained from cell lines containing pPAM, pPAM2, and pPAM3; however, cell lines made with pPAM4 were about 10-fold less efficient in packaging HPRT virus (Table 1). Since virus titer is critical for many applications of retroviral vectors, we used the best pPAM3 transfectant, PA317, in further studies instead of the best pPAM4 transfectant, PA405. The deletions made in pPAM3 should be sufficient to severely reduce packaging of RNA derived from this construct into virions, and even if the RNA is packaged, provirus formation in infected cells should be blocked both at the level of reverse transcription of the RNA to DNA and at the level of virus integration into the host genome.

Comparison of virus titers from vector-infected packaging cell lines. The titers of virus produced transiently following transfection of PA12 or PA317 cells with HPRT virus were similar, indicating that PA317 cells efficiently package retrovirus vectors, as do PA12 cells. To further test the PA317 line, we infected PA317 cells with a virus containing a dominant-acting, selectable DHFR gene (Fig. 2), assayed individual infected clones for production of DHFR virus, and compared these results with those obtained using PA12 cells (Table 2). The DHFR virus titer produced from DHFR virus-infected PA317 clones was very high, up to 10^7 DHFR virus per ml of medium with no detectable helper virus. The PA317 clones on average produced slightly higher-titer DHFR virus than the PA12 clones, and the best clone was slightly better than the best PA12 clone. We concluded that the additional mutations present in the packaging DNA in PA317 cells did not adversely affect our ability to make high-titer-vector-producing cell lines.

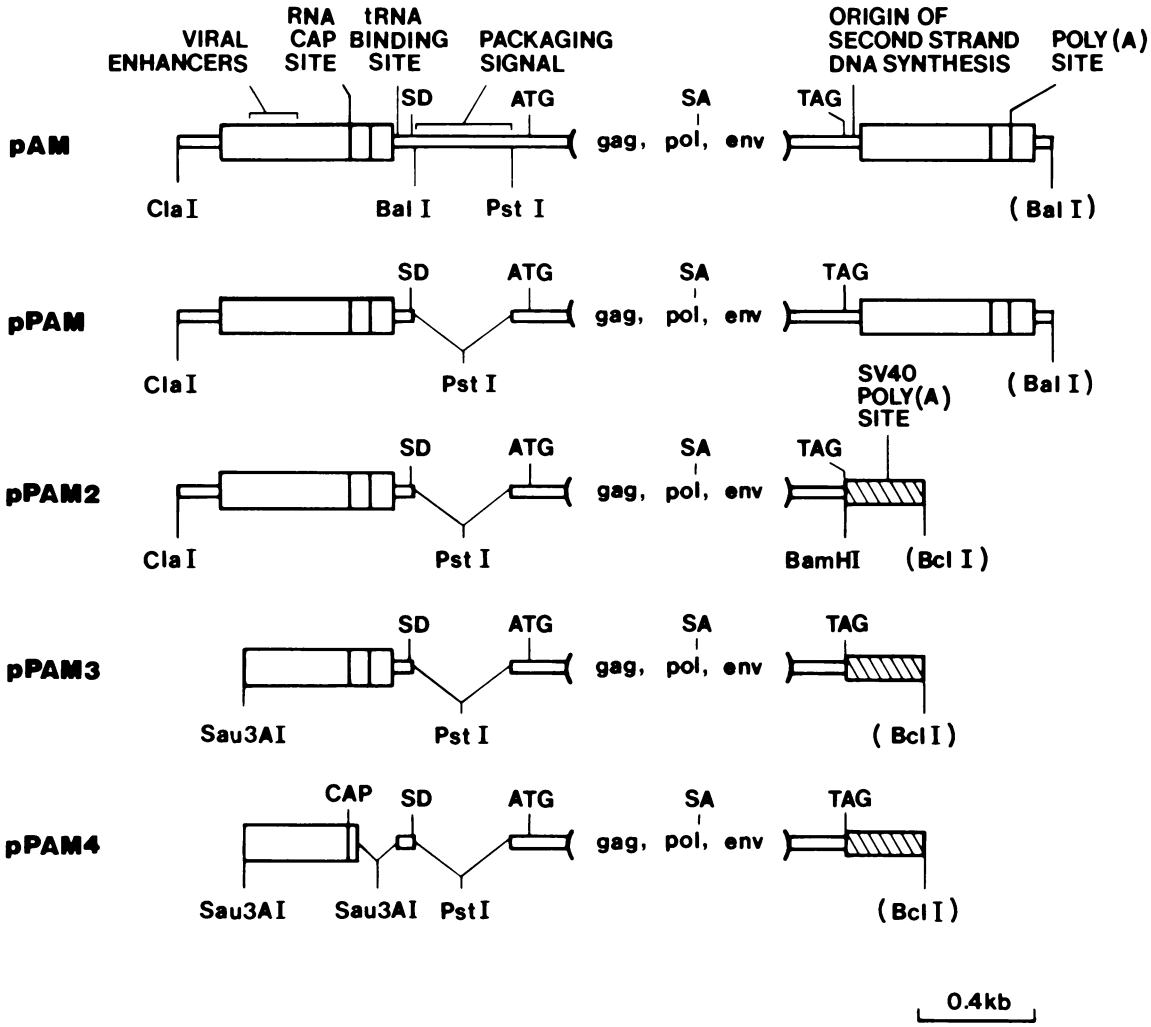

FIG. 1. Packaging constructs are depicted without surrounding plasmid sequences. Large, open boxes represent retroviral LTRs; small, open boxes represent other retroviral sequences; and hatched boxes denote simian virus 40 sequences. Landmark restriction sites are shown, but all of the sites for a given enzyme are not necessarily shown. The plasmid containing a recombinant amphotropic helper virus (pAM) and the derivative of this virus in which the packaging signal has been removed (pPAM) have been described before (15). Important features for retrovirus replication are noted above the pAM construct. The construct pPAM2 was made from pPAM by replacement of the 3' LTR with the late polyadenylation signal from simian virus 40, isolated as a 237-base-pair *Bam*HI to *Bcl*I fragment. The end of the retroviral genome was cleaved with *Rsa*I at position 7762 (25), which cuts just upstream of the termination codon of *env*, and a synthetic oligonucleotide was added to duplicate the part of *env* that was removed. This oligonucleotide also contained a *Bam*HI site downstream of the terminator codon for *env* for addition of the simian virus 40 fragment containing a polyadenylation signal. pPAM3 was made from pPAM2 by removing viral sequences 5' of the viral enhancers in the 5' LTR by using an *Sau*3AI site at position −352 (25). In addition to the deletions in pPAM3, pPAM4 has a deletion which removes the tRNA-binding site and 3' portion of the 5' LTR while preserving the splice donor site. This deletion was made by cleavage of the LTR at an *Sma*I site at position 28 (25) in the R region of the LTR, addition of a *Bam*HI linker, and linkage to an *Sau*3AI site at position 161 (25) (*Bam*HI and *Sau*3AI leave complementary 5' extensions). The constructs are all carried in pBR322. pAM, pPAM, and pPAM2 are inserted in pBR322 in place of the Tc^r gene between the *Cla*I and *Pvu*II sites. The *Cla*I sites remain, but the sites at the other ends of the constructs were destroyed during attachment to the *Pvu*II site of pBR322. pPAM3 and pPAM4 were inserted in place of the Tc^r gene between *Bam*HI and *Pvu*II. Only the *Sau*3AI site remains at the 5' end of the construct, and the sites at the 3' end have been destroyed. kb, Kilobase; SD, splice donor; SA, splice acceptor.

Inhibition of recombination to produce helper virus. We have previously shown that certain vectors interact with PA12 cells to produce helper virus (16). N2 virus (Fig. 2) and derivatives containing additional genes inserted at the 3' end of the virus produce helper virus when introduced into PA12 cells. Helper virus production is dependent on the presence of *gag* sequences upstream of the *neo* gene in this vector, as deletion of these sequences leads to vectors that do not produce helper virus (16). A plausible model for this event would involve copackaging of the viral vector and RNA derived from the pPAM packaging construct present in PA12 cells, followed by recombination in the *gag* region during reverse transcription in an infected cell. Addition of the 5' portion of the vector to the packaging construct would result in creation of a fully replication-competent virus containing a vector-derived packaging signal. In contrast, two recombination events would be required to generate helper virus from the pPAM3 packaging construct, since defects are

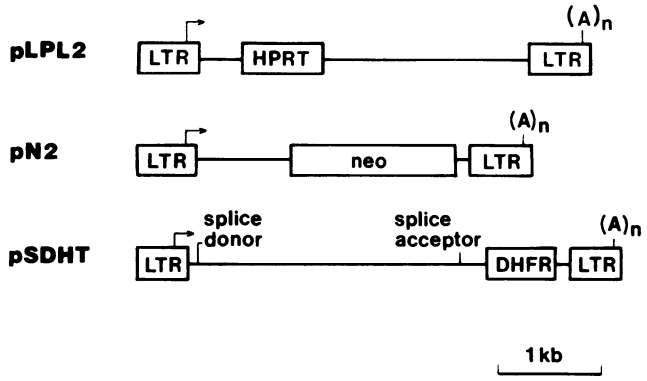

FIG. 2. Viral vectors. Selectable markers in these vectors were expressed by using transcriptional signals present in the viral LTRs. The Neo virus pN2 (11) and the DHFR virus pSDHT (16) have been described before. The HPRT virus pLPL2 is similar to the pLPL virus described previously (14) with two modifications. First, two GC-rich regions in the HPRT cDNA (10) that lie between the 5′ LTR and the start codon of HPRT in pLPL were removed. *Nae*I was used to cut the HPRT cDNA 9 base pairs 5′ of the start codon (10), and a *Pst*I linker was added, cleaved with *Pst*I, and joined to the *Pst*I site present in pLPL at the start of the HPRT cDNA. Second, in addition to the normal retroviral packaging signal between the 5′ LTR and HPRT, a second packaging signal lies downstream of the 3′ LTR in pLPL, and this signal might allow packaging of RNAs initiating in the 3′ LTR and extending into pBR322 sequences following transfection of pLPL into packaging cell lines. To avoid this possibility, the second signal was removed by cleavage at a *Bal*I site just 3′ of the 3′ LTR, and an *Eco*RI to *Bal*I fragment containing the HPRT virus was inserted in place of the Tet^r gene of pBR322 between the *Eco*RI and *Pvu*II sites. HPRT virus production following transfection of pLPL2 into packaging cells was about threefold higher than that of pLPL. kb, Kilobase.

present at both ends of pPAM3. A double recombination event is presumably quite rare; thus, it might be possible to generate helper-free virus by introduction of the N2 vector into PA317 cells.

This prediction was confirmed in the following experiment. The N2 virus was introduced into PA12 or PA317 retrovirus packaging cells, and independent clones containing the virus were isolated and screened for the production of Neo virus and helper virus. All of the PA12-N2 clones produced Neo virus, and all but one produced helper virus (Table 3). Helper virus production by this clone was detected after further passage of the cells. The entire experiment was repeated with a similar result. Similarly, most of the PA317-N2 clones produced Neo virus, but in contrast none of the clones produced helper virus (Table 3). Passage

TABLE 1. Ability of clonal cell lines cotransfected with packaging constructs to package a retroviral vector (pLPL2)[a]

Packaging construct	Highest virus titer (CFU/ml)	% of clones producing >20% of maximum titer	% of clones producing no virus (<10 CFU/ml)	Best clone
pPAM	3×10^4	50	13	PA12
pPAM2	6×10^4	40	40	PA212
pPAM3	6×10^4	40	29	PA317
pPAM4	4×10^3	25	50	PA405

[a] Clones were isolated from two separate transfections. At least eight clones from each transfection were screened.

TABLE 2. Virus production from packaging cells containing SDHT DHFR virus[a]

Packaging cell line	SDHT virus-infected cell clone	DHFR virus titer (CFU/ml)	Helper titer (FFU/ml)[b]	DHFR virus titer after helper virus infection (CFU/ml)
PA12	1	2×10^6	<1	
	2	2×10^6	<1	
	3	2×10^3	ND[c]	
	4	1×10^4	ND	
	5	4×10^4	ND	
	6	4×10^6	<1	
PA317	1	6×10^5	<1	6×10^6
	2	6×10^5	<1	4×10^6
	3	1×10^7	<1	3×10^7
	4	4×10^6	<1	
	6	3×10^5	<1	6×10^6
	7	$<1 \times 10^3$	<1	4×10^7
	8	5×10^6	<1	
	9	1×10^5	<1	10^6

[a] Packaging cell lines containing retroviral vectors were made by transfecting Psi-2 cells followed by harvesting of virus 2 days later and infection of amphotropic packaging cell lines as previously described (16). Independent vector-infected cell lines were isolated following drug selection by using cloning rings.
[b] FFU, Focus-forming units.
[c] ND, Not determined.

of two of the high-titer Neo virus-producing cell lines for 1 month did not result in helper virus production, as measured by the S^+L^- assay. In addition, NIH 3T3 cells infected with 1-ml samples of virus from these lines did not produce helper virus or Neo virus when assayed more than 2 weeks after infection. Thus, helper virus production from PA317 cells containing N2 virus was not detected, whereas production from PA12 cells containing N2 virus was always detected.

Previous reports indicate that when N2 virus was

TABLE 3. Virus production from packaging cells containing N2 Neo virus[a]

Packaging cell line	N2 virus-infected cell clone	Neo virus titer (CFU/ml)	Helper titer (FFU/ml)	Neo virus titer after helper virus infection (CFU/ml)
PA12	1	5×10^6	20	
	2	4×10^6	<1	
	3	2×10^7	40	
	4	1×10^7	190	
	5	5×10^6	200	
PA317	1	8×10^6	<1	2×10^7
	2	1×10^6	<1	
	3	2×10^3	<1	3×10^7
	4	4×10^6	<1	
	5	2×10^6	<1	
	6	1×10^5	<1	4×10^7
	7	4×10^5	<1	1×10^7
	8	6×10^5	<1	
	10	2×10^5	<1	
	11	1×10^7	<1	
	Population[c]	6×10^6	<1	

[a] For experimental conditions, see Table 2, footnote *a*.
[b] FFU, Focus-forming units.
[c] A population of over 100 independent clones was analyzed to check for rare events leading to helper virus production.

transfected into Psi-2 cells, helper virus production from clonal cell lines was not detected (6, 11). Psi-2 cells contain a packaging construct which is similar to that of PA12 cells except that the envelope regions are from murine viruses with different host ranges. Thus, one might predict that Psi-2 cells would interact with N2 virus to generate helper virus in a fashion analogous to that of PA12 cells. We tested for production of helper virus from Psi-2 cells after introduction of N2 virus by using the XC helper virus assay (21). This assay is used for detection of Moloney murine leukemia virus, the virus on which the Psi-2 line is based. In addition to isolating clonal cell lines containing N2 virus, we analyzed cell populations containing many independent clones so that a rare event resulting in helper virus production might be detected. These populations of cells were made by transfection of Psi-2 cells with pN2 or by infecting the cells with helper-free virus from the cell line PA317-N2 c11 (Table 3). All of the populations of Psi-2 cells containing N2 virus secreted helper virus in addition to N2 virus (Table 4). In addition, three of the six clonal N2-transfected Psi-2 cell lines secreted helper virus in addition to N2 virus. The level of helper virus secretion was highly variable among the cells analyzed, which may reflect a relatively infrequent event which generates helper virus followed by slow spread of helper virus in the cells. In contrast, we did not detect helper virus production from Psi-2 cells not containing vectors. Thus, even though it may be possible to isolate clones of N2 virus-containing Psi-2 cells which do not secrete helper virus, production of helper virus is a frequent event.

Analysis of packaging clones that produce low-titer virus. Some of the PA317 cell clones infected with selectable vectors produced low-titer virus, for example, PA317-SDHT c7 (Table 2) or PA317-N2 c3 (Table 3). The explanation for this might be that the integrated virus suffered mutations so that it could not efficiently replicate or that PA317 cells are heterogeneous in their ability to express virus-packaging functions. To answer this question, we infected several PA317-SDHT and PA317-N2 clones with AM-MLV helper virus, passaged the cells for over 2 weeks to allow the helper virus to spread, and assayed the clones for production of virus. After infection, all of the clones analyzed produced high vector titers (Tables 2 and 3), showing that the integrated SDHT and N2 vectors in these cells were not defective. We therefore concluded that there is heterogeneity in the packaging ability of PA317 cells, possibly because of loss of the transfected pPAM3 DNA that is required for retrovirus vector packaging.

Sensitive assay for helper virus production. We next attempted to increase the sensitivity of the S^+L^- assay in an attempt to detect helper virus production from PA12 or PA317 cells. In many experiments performed in this laboratory we have never seen helper virus production from PA12 cells as measured by the S^+L^- assay. We have detected helper virus production from retrovirus vector-containing cells only in the case of N2 Neo virus and its derivatives. However, an attempt to increase the sensitivity of the assay might result in helper detection or detection of a rare event involving transfer of the packaging function.

The S^+L^- helper virus assay involves exposure of cat cells harboring a replication-defective transforming virus to test virus, followed by overlay of the cat cells with nontransformed rat NRK cells. The presence of helper virus in the test virus is indicated by focus formation in the otherwise flat NRK cells, owing to rescue of the transforming virus followed by infection and transformation of the NRK cells. To increase the sensitivity of the assay, we repeatedly exposed cat cells at 12-h intervals to large quantities of medium harvested from cells to be tested for helper virus production and then cocultivated the cat cells with NRK cells. The assay was performed using PA12 and PA317 cells and DHFR virus-producing PA12 and PA317 cells (PA12-SDHT c6 and PA317-SDHT c3 [Table 2]). No foci were induced by any of these cell lines; thus, we were unable to detect helper virus production by these lines (<0.1 helper virus per ml) (data not shown). In contrast, AM-MLV helper virus harvested from AM-MLV virus-producing NIH 3T3 TK^- cells induced 3×10^6 foci per ml of medium. In addition, critical observation revealed no differences between the test plates and a mock-infected control dish; thus, there was no indication in this assay of transfer of the packaging function. Transfer of the packaging function into cat cells should have resulted in production of transforming virus, but the rate of production may have been too low to result in focus induction in NRK cells, or perhaps helper virus-mediated spread of transforming virus is required for focus formation.

TABLE 4. Virus production from Psi-2 cells containing N2 Neo virus[a]

N2 virus-transfected Psi-2 cell clone[a]	Neo virus titer (CFU/ml)	Helper titer (PFU/ml)[b]
1	2×10^4	<1
2	9×10^4	<1
3	8×10^5	10^2
4	4×10^5	$>10^3$
5	2×10^4	$>10^3$
6	3×10^5	<1
Population a	$>10^4$	2×10^2
Population b	$>10^4$	8
Population c	$>10^4$	5×10^2
Population d	$>10^4$	$>10^5$

[a] Cell populations a and b were derived by infection of Psi-2 cells with helper-free virus from PA317-N2 c11 cells. Cell populations c and d were derived by transfection of Psi-2 cells with pN2 without cloning the resultant colonies.

[b] Helper virus was measured by using the XC assay as previously described (17).

Detection of packaging function transfer. We designed the experiment depicted in Fig. 3 as a sensitive assay for detection of packaging function transfer. First, NIH 3T3 (TK^-) cells harboring but not producing a Neo virus (NIH 3T3-N2 cells) were exposed to medium harvested from various packaging cells. If the NIH 3T3-N2 cells became infected by virus, resulting in transfer of the packaging function, they would begin to secrete Neo virus. We next mixed the infected NIH 3T3-N2 cells with rat $HPRT^-$ fibroblasts, cocultivated the cells for 4 days, and assayed the mixed populations for the presence of $HPRT^-$, Neo virus-infected rat cells by exposing the cells to G-418 and 6-thioguanine and scoring resistant colonies. 6-Thioguanine kills $HPRT^+$ cells, so the NIH 3T3-N2 cells were killed, and G-418 kills rat cells unless they are infected with Neo virus. Colony formation was thus indicative of rescue of the Neo virus by virus produced by the packaging cells. The reason for the cocultivation step in this assay was that small amounts of virus produced by a few NIH 3T3-N2 cells were not detected in medium exposed to the cells (data not shown), presumably because the virus bound rapidly to cells in the dish which were not producing virus and thus had available viral receptors. Cocultivation of NIH 3T3-N2 cells

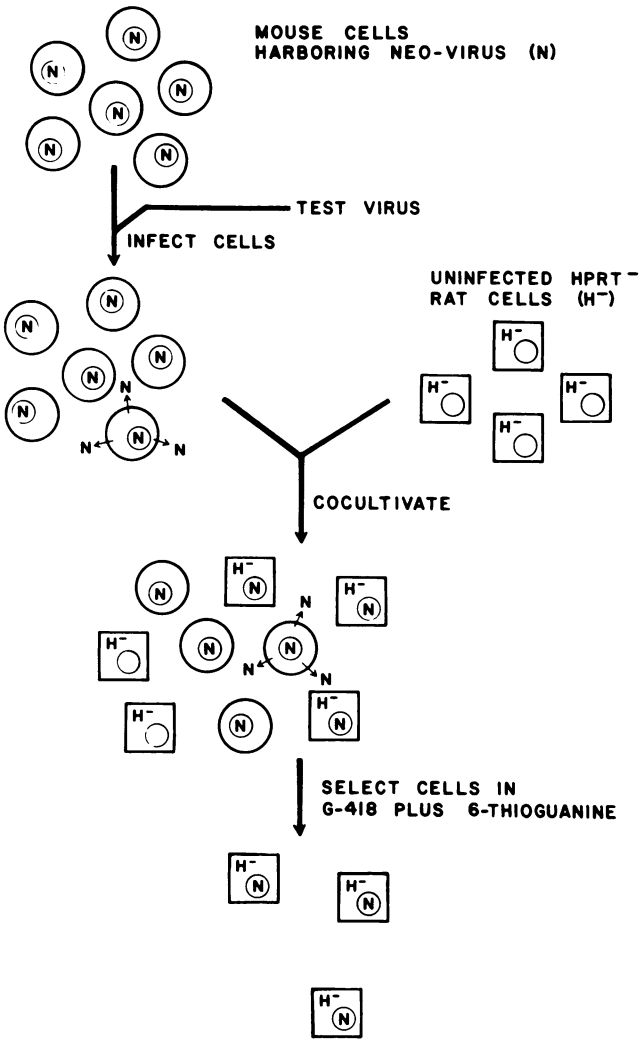

FIG. 3. Scheme for assay of packaging system transfer.

with rat cells allowed transfer to occur directly between cells and therefore increased the sensitivity of the assay.

Using this cocultivation assay, we found that medium from Psi-2 cells consistently induced low-level Neo virus release from NIH 3T3-N2 cells (Table 5). Medium from PA12 cells or PA12 cells producing DHFR virus also induced Neo virus release but at even lower levels. We did not detect colony production using medium from PA317 cells or DHFR virus-producing PA317 cells. We ruled out endogenous mouse viruses as being responsible for Neo virus rescue in these experiments because these viral genomes were present in all of the packaging lines, whereas not all induced Neo virus production. Retroviruses can induce cell fusion, but colony formation as a result of cell fusion was ruled out because fusion products between the HPRT$^-$ rat and G-418-resistant mouse cells would still be sensitive to 6-thioguanine, since the mouse cells were HPRT$^+$. In addition, we ruled out colony formation as a result of cell fusion because virus from the packaging cell lines should induce fusion at similar rates, and some lines did not induce colony formation. We concluded that deletion of the packaging signal was not sufficient to prevent transfer of the packaging function. However, additional alterations in the packaging system in PA317 cells reduced packaging function transfer to undetectable levels.

DISCUSSION

We made a systematic series of deletions in a replication-competent retrovirus in an attempt to make a nontransmissible virus which still provides *trans*-acting factors required for packaging and transmission of retroviral vectors. These deleted constructs were introduced into cells by cotransfection with a selectable marker to make various retrovirus packaging cell lines. Packaging cells made using the most heavily deleted construct, pPAM4, did not produce virus vectors at high titer. Packaging cell lines made using an intermediate construct, pPAM3, did produce virus vectors at high titer (10^6 to 10^7 CFU/ml). The packaging construct pPAM3 was made by deletion of the retroviral packaging signal, all of the 3′ LTR, part of the 5′ LTR, and the site for second-strand DNA synthesis. Thus, RNA transcribed from pPAM3 should have been inefficiently packaged into virions, and reverse transcription and integration of any resultant virus should have been impossible. Indeed, we were unable to detect packaging function transfer from PA317 cells containing pPAM3.

Others have attempted to make packaging lines analogous to PA317 cells. A packaging cell line that contains a virus with deletions of the origin of second-strand DNA synthesis as well as the packaging signal has been previously described (24), but vector titers from this line (10^3 CFU/ml) are low. A packaging system with a limited host range that is based on avian reticuloendotheliosis virus has been described before (27). The *gag* and *pol* proteins on the one hand and the *env* protein on the other are synthesized from separate DNA constructs. The packaging signal was also removed from both constructs. However, helper virus production or packaging system transfer from this line was not extensively analyzed.

We did not observe helper virus production from cells transfected with any of the packaging constructs. This includes the construct pPAM, in which only the retroviral packaging signal was removed. This is similar to results obtained by other groups using constructs similar to pPAM (3, 24). In experiments leading to construction of the Psi-2 packaging cell line (13), helper virus production from the packaging signal-deleted helper virus used was very frequent; however, a later report suggested that this was probably due to the presence of a small amount of wild-type helper virus plasmid in the construct plasmid stock, as other preparations of the construct did not lead to helper virus production (12). In addition, we have never observed helper virus production from PA12 cells carried in the laboratory for many months, as measured by S$^+$L$^-$ assay of the cells and their derivatives containing various retroviral vectors,

TABLE 5. Detection of Neo virus rescue from cells following infection with medium exposed to packaging cell lines

Test cells	No. of colonies induced	
	Expt 1	Expt 2
NIH 3T3 (TK$^-$)	0	0
Psi-2	18	10
PA12	3	2
PA12-SDHT	0	1
PA317	0	0
PA317-SDHT	0	0

with the exception of PA12 cells containing the Neo virus pN2. Neither have we observed helper virus production from Psi-2 cells, except after introduction of the N2 vector. However, we know of two examples from other laboratories in which helper virus arose in PA12 cells in the absence of any apparent source of contamination. We confirmed the presence of helper virus in one case (unpublished data). Perhaps at very low frequency helper virus production can occur, possibly as a result of recombination of the packaging system with endogenous retrovirus like DNA or RNA elements found in mouse cells. This phenomenon should be significantly reduced in the new packaging cell lines described here.

The Neo virus N2 interacted with PA12 or Psi-2 packaging cells to yield helper virus at high frequency. We hypothesize that this is due to copackaging of packaging system RNA and Neo virus RNA, followed by recombination in the common *gag* region during reverse trancription in an infected cell. This event occurs in packaging cell populations, as clonal lines can be isolated that are initially helper free but produce helper virus eventually. Whereas cells infected with replication-competent virus are resistant to reinfection with virions having the same pseudotype, PA12 and Psi-2 cells are much less resistant (16); thus, infection and recombination between copackaged RNAs can occur in these packaging cell lines. In contrast, we did not detect helper virus production from PA317 cells containing N2 virus. A double recombination event would be required between the packaging system and N2 virus in this case, which must be rare. N2 virus has characteristics which make it a useful vector. For instance, in canine and human marrow infections, only N2 and another virus containing *gag* sequences permitted efficient infection (9, 11a). The PA317 cell line now permits production of N2 virus in the absence of helper virus, and this utility probably extends to other potentially useful vectors which interact with previously available packaging cell lines to produce helper virus.

Retroviral vectors produced by PA317 cells could infect mouse, rat, cat, dog, and human cells (data not shown), and thus they have an amphotropic host range. In particular, we were able to infect hemopoietic progenitor cells from human bone marrow using retroviral vectors secreted from PA317 cells (R. A. Hock and A. D. Miller, unpublished data). Vector titer from the cells was also very high (up to 10^7 CFU/ml); thus, these cells should be useful in experiments aimed toward human gene therapy.

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